The Release of Oil from Petroleum-Bearing Materials by Sulfate-Reducing Bacteria

D. M. UPDEGRAFF AND GLORIA B. WREN[1]

Field Research Laboratories, Magnolia Petroleum Company, Dallas, Texas

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The possible importance of bacteria in relation to the production of petroleum from underground reservoirs has been recognized in recent years. A development which did much to arouse the petroleum industry's interest in microbiology was the announcement, by ZoBell and co-workers, of a bacteriological treatment which in laboratory tests appeared to promote the release of oil from oil-bearing sands and shales (ZoBell, 1946a, 1946b, 1947a, 1947b). The work on which the announcement was based was carried on as a part of a research project on the role of microorganisms in petroleum genesis. This project was financed by a grant from the American Petroleum Institute. The patent issued on the process (ZoBell, 1946b) therefore was dedicated to the public without charge.

The accidental discovery of the process was a by-product of the project's work on the oxidation of hydrocarbons in oil sands by cultures of *Desulfovibrio* bacteria. The simple apparatus used was a glass-stoppered bottle containing some oil sand and filled with a saline aqueous medium. Such bottles reportedly showed migration of more oil from the sand to the surface of the medium when bacteria were present than when bacteria were excluded. Lactate was added to the aqueous medium in the bottles in order to provide a satisfactory energy source for the bacteria. Growth of *Desulfovibrio* was negligible in the absence of such added nutrients. ZoBell's work on oil release was discontinued after completion of the patent application in 1944.

In his papers on the subject cited above, ZoBell suggested several mechanisms which could be responsible for the promotion of oil migration or release by bacteria, namely:

1. Dissolution of limestone or other calcareous cementing or intergranular materials associated with the porous rock in which petroleum is found, and decomposition of sulfate minerals by these bacteria or the acids produced by them, resulting in improved permeability of the rock and probable improved oil recovery.

2. Production of carbon dioxide, methane, and hydrogen by bacteria which promote the flow of oil by two mechanisms; reduction of the viscosity of the oil by solution of gas in the oil and increase in gas pressure.

3. Production of detergents by the bacteria (perhaps by oxidation of hydrocarbons in the oil) which help remove the oil.

4. Tenacious adherence of the bacteria to solid surfaces which tends to displace the oil from the sand surfaces.

5. Reduction of the viscosity of the oil by direct chemical action of the bacteria upon the oil.

ZoBell's patent claims the method of treating a fluid-bearing earth formation to increase the recovery of fluids therefrom by subjecting the formation to the action of *Desulfovibrio hydrocarbonoclasticus* or *Desulfovibrio halohydrocarbonoclasticus*. These are two new species of sulfate-reducing bacteria. The first would fall under the species *D. desulfuricans* or *D. rubentschickii* and the second under *D. aestuarii* of Breed *et al.* (1948) who recognize these three as the only established species in this genus. The two species named by ZoBell differ from the recognized species in their alleged ability to oxidize hydrocarbons. There is no evidence that sulfate-reducing bacteria other than the hydrocarbon-oxidizing strains can carry on activities which would be effective in releasing oil.

Beck (1947) carried out extensive experiments, with ZoBell's bottle culture method, and also with consolidated sandstone cores. Inconsistent results were obtained and no clear demonstration of bacterial oil release was observed. Evidence for the utilization of Bradford, Pennsylvania, crude oil by sulfate-reducing bacteria, including many hydrocarbon-oxidizing strains obtained from ZoBell, was so meager and inconsistent that Beck concluded that "utilization of crude oil by these bacteria under field conditions would be negligible". O'Bryan and Ling (1949) found that sulfate-reducing bacteria, when injected in a nutrient medium into cores of Edwards limestone, partially plugged the cores as shown by a decline in permeability. Mackenzie (1952) reported that sulfate-reducing bacteria utilized crude oil, and that some evidence of oil release was obtained. He emphasized the importance of phosphate as an essential mineral nutrient for the bacteria. Unfortunately, his paper was published in abstract form only, and it is therefore impossible to evaluate his data.

[1] Present address: Mrs. Donald B. Wren, 12th Air Force Headquarters, Section A1, APO 12, % Postmaster, New York City, N. Y.

Kuznetsov (1950) reported the isolation of sulfate-reducing bacteria from core samples taken from depths up to 1618 meters. He found that sulfate reducing bacteria utilized only one of three different types of Russian crude oil tested, and drew the important conclusion that the reduction of sulfates at the expense of the organic matter in petroleum proceeds extremely slowly and depends on the chemical composition of the petroleum. ZoBell (1953) has extended his patent coverage to the use of hydrogen-oxidizing sulfate-reducing bacteria. Updegraff and Wren (1953), recognizing the fact that sulfate-reducing bacteria multiply extremely slowly when forced to subsist upon crude oil as a sole energy source, obtained a patent on the injection of molasses into petroleum reservoirs in order to stimulate their growth rate. The data upon which this patent application was based relied upon comparisons with sterile controls containing mercuric chloride, a procedure later found to give erroneous results. Packs treated with molasses medium and sulfate-reducing bacteria gave better oil recovery than those treated with other media, but the mass of data gathered since indicates that the methods used in the earlier experiments contained random errors which now make it impossible to verify such a conclusion.

Our studies were performed in order to evaluate the method from the standpoint of its usefulness in increasing the recovery of oil from petroleum reservoirs. The approach was concerned primarily with the most fundamental requirement of any proposed oil recovery method, that is the demonstration of whether the process has any favorable effect on the rate and/or amount of oil recovery from porous media. The mechanisms advanced by ZoBell to explain oil release by sulfate-reducing bacteria were also given some investigation.

Materials and Methods

Cultures Used

A total of 38 enrichment cultures of sulfate-reducing bacteria were employed in this study. They comprised 5 cultures of *Desulfovibrio halohydrocarbonoclasticus*, obtained from Dr. C. E. ZoBell, and numerous cultures of *Desulfovibrio* isolated by us from soil, marine sediments, and brines produced from oil wells. Enrichment cultures were employed instead of pure cultures for several reasons. First it would be difficult, if not impossible, to maintain pure cultures in any field application of a bacterial oil recovery method. Second, sulfate-reducing bacteria often grow better in enrichment culture than in pure culture, and third, enrichment cultures can be subcultured indefinitely without any marked changes in characteristics, whereas pure cultures often die out or become contaminated upon subculture.

Media

Most of the media are based on a formula, medium C, resembling sea water in ionic composition, but enriched in phosphate, ammonium, and iron. Media M, M1, M2, and M3 are based on the medium found by Miller (1949) to give the maximum rate and amount of sulfate reduction by *Desulfovibrio* cultures from either fresh or salt water environments. The composition of each medium used is given below:

1. *Medium C*

NaCl	20 g
$MgSO_4$	0.5 g
Na_2CO_3	0.1 g
H_3BO_3	0.03 g
$FeSO_4 \cdot 7H_2O$	0.03 g
$(NH_4)_2SO_4$	1.0 g
KH_2PO_4	0.1 g
Distilled water	to 1000 ml

pH 7.2–7.6

2. *Medium A*
   Same formula as Medium C with the addition of 2.0 g per liter of calcium lactate.
3. *Medium AY*
   Same formula as Medium A with the addition of 2.0 g per liter of Difco yeast extract.
4. *Medium AX*
   Same formula as Medium A with the addition of 0.5 g per liter of Difco yeast extract.
5. *Medium CM*
   Medium C with the addition of 0.1 to 0.4 per cent blackstrap molasses as follows:
   CM-1, 0.1 per cent molasses
   CM-2, 0.2 per cent molasses
   CM-4, 0.4 per cent molasses
6. *Medium CX*
   Same as Medium C, but with 0.5 g per liter of Difco yeast extract added.
7. *Medium CY*
   Same as Medium C, but with 2.0 g per liter of Difco yeast extract added.
8. *Medium M*

NaCl	10.0 g
$MgSO_4$	0.90 g
$CaCO_3$	1.00 g
$FeSO_4 \cdot 7H_2O$	0.01 g
$CaCl_2 \cdot 2H_2O$	0.10 g
Na_2SO_4	18.25 g
NH_4Cl	1.00 g
K_2HPO_4	0.50 g
Sodium lactate	21.0 g
Distilled water	to 1000 ml

pH 7.2–7.6

9. *Medium M1*
   Same as above, but omitting sodium lactate and adding 1 g per liter of yeast extract.
10. *Medium M2*
    Same as medium M, but omitting sodium lactate and adding 0.5 g per liter of yeast extract and 0.06 g per liter of reduced iron powder.
11. *Medium M3*
    Same as medium M2, but without iron powder, and with 10 ml per liter of 1/5,000 methylene blue and 5 ml per

liter of sterile 1 per cent Na_2S solution added after autoclaving.

Preparation of Unconsolidated Sand Packs

Clean, dry sand or other material was sieved, and the desired size fraction collected. The collected material was then washed with distilled water to remove clays and soluble matter and dried and sterilized by heating to 182 to 560 C overnight, cooled, and packed into glass tubes. A few glass beads and a small wad of glass wool served to retain the sand in the bottom end of the glass tubes. Glass tubes from ⅞ to 1⅜ inches in diameter and from 9½ to 15 inches long have been used. Perforated porcelain discs were used as retainers in the upper end. Retainers for Lucite-tube sand packs were perforated Lucite discs, and for Saran pipe were stainless steel screens. The dimensions of the tubes, and other data, are given in table 16, p. 320.

Carbon dioxide gas was flowed through the prepared packs to dilute and remove air, and this was followed by a flood with the aqueous medium. The oil was then flowed into the vertical pack (usually vertically downward) to displace the aqueous medium. This was conducted until no water was produced. From 10 to 100 pore volumes of aqueous medium then were passed vertically upward through the pack at 0.5 to 2 psi differential pressure to displace oil. This was continued until no more oil was produced. In a given experiment using the same oil, sand, and apparatus, the residual oil saturations after flooding with the aqueous medium sometimes varied widely. Later improvements in procedure and technique resulted in improved reproducibility in values of residual oil saturation.

Either the inoculum (a culture of the desired bacteria) or a germicide solution was then introduced into one end of each sand pack, and the packs were set aside to incubate to allow time for the bacteria to multiply.

After 7 to 100 days' incubation at room temperature, or at a constant temperature of either 30 C or 37 C, the packs were flooded out with 50 to 100 pore volumes of the same sterile aqueous medium with which the packs were initially saturated, and the additional released oil was measured in a burette or graduated cylinder. Residual oil was calculated from the difference between the initial oil content of the pack and the total released oil.

Mercuric chloride initially was used as a germicide in the control experiments used to evaluate the effects of bacteria on oil recovery. Ten such experiments were carried out with packs of sand or crushed limestone material, and one with a plug cut from a dolomite core. It was found that mercuric chloride retards oil recovery by water flooding, resulting in less oil release and a higher residual oil content than would have been obtained by water flooding in the absence of mercuric chloride. The data of table 1 illustrate this effect. However, during the course of these experiments much was learned regarding experimental techniques for measuring oil release by bacterial action.

The oil-recovery retarding action of mercuric chloride is attributed to the fact that it forms insoluble salts with the sulfur compounds found in crude oil (Faragher *et al.*, 1929). When the crude oils used in the above study were shaken with an aqueous 1 per cent solution of mercuric chloride a voluminous, gummy precipitate formed at the interface between the oil phase and the aqueous phase. The experiments employing mercuric chloride therefore do not furnish any evidence regarding bacterial oil release, and will not be discussed further.

Details on the apparatus and materials used in the various oil recovery experiments are given in the data tables for the individual experiments (tables 1 to 11 inclusive) and in table 16.

Hydrocarbon Utilization

Methods used for the experiments on the utilization of crude oil and hydrocarbons by *Desulfovibrio* included many variations. Basically, the method involved was to grow the cultures in a mineral salts medium containing crude oil or n-hexadecane as the sole energy source, or in a similar medium with a small amount of yeast extract added as a growth stimulant. Hydrogen sulfide was then determined on each culture by potentiometric titration in 2-normal ammonium hydroxide with 0.05-normal silver nitrate and a silver electrode. This method, a modification of that of Tamele and Ryland (1936), has proved to be, in our experience, more sensitive and specific than the iodimetric method for the determination of sulfide.

Results

Oil Release Measurements on Sand Packs under Static Conditions

The data from these experiments are set forth in tables 1 to 8. In every pack inoculated with *Desulfovibrio* the organisms grew vigorously.

By observing the rate at which the blackened zone (the color resulted from precipitation of FeS in the pack by bacterially-produced H_2S) moved through the packs, it was established that the organisms advanced in the absence of flow at a rate ranging from 0.5 to 1.5 inches per day at 30 C. Cultural counts on the long sand pack (table 8) showed that the *Desulfovibrio* bacteria moved at the rate of 1.0 inches per day in this pack at room temperature (20 to 27 C). This movement is partly due to their multiplication and partly to their motility.

In experiment 2 (table 2) all of the 6 controls were heat sterilized. Unfortunately none of them remained sterile throughout the incubation period; two of the

TABLE 1. *The effect of mercuric chloride on oil recoveries from static sand packs incubated for 66 days at 30 C*

Pack No.	Content of Sand Packs		Oil as Per Cent of Pore Volume		
	Sand wet with	Final content	Initial	Released	Residual
10	Medium CM-1	Saturated mercuric chloride—sterile	37	3.7	33
2	Medium CM-1	Sterile medium	31	13	17
3	Tap water	Sterile tap water	16	6.2	10
9	Tap water	Tap water—inoculated	19	4.0	15
11	Medium CM-1	Medium—inoculated	33	15	18
6	Tap water	Saturated mercuric chloride—sterile	26	5.0	21
12	Medium CM-1	Medium + 1 per cent mercuric chloride inoculated	34	9.5	25

Sand: 35–45 mesh Ottawa. Oil: Belridge, California, crude, 14° API.

TABLE 2. *Oil recoveries from small static sand packs incubated for 80 days at 30 C*

Pack No.	Contents of Pack	Oil as Per Cent of Pore Volume		
		Initial	Released	Residual
1	Medium C—inoculated	38	19	19
2	Medium C—sterile	38	19	19
3	Medium C without sulfate inoculated	34	17	17
4	Medium C without sulfate sterile	33	19	14
5	Medium CM-1—inoculated	35	11	24
6	Medium CM-1—sterile	33	12	21
7	Medium C without sulfate + 1 per cent yeast—inoculated	22	13	9
8	Medium C without sulfate + 0.1 per cent yeast—sterile	28	12	16
9	Medium C + 0.1 per cent calcium lactate—inoculated	30	14	16
10	Medium C + 0.1 per cent calcium lactate—sterile	32	14	18
11	Medium CM-1—inoculated	33	8	25
12	Medium CM-1—sterile	30	7	23

Oil: Wilmington, California, crude, 15° API. Sand: 35–45 mesh Ottawa.

6 controls became contaminated with *Desulfovibrio*, and the other 4 with other types of microorganisms.

Examination of the data reveals close agreement in released and residual oil percentages obtained from the 2 packs of a pair treated with the same flooding medium, except for the pair comprising packs 7 and 8. On the other hand, there were rather large differences in residual oil content between packs flooded with media of different chemical composition. This suggests that small amounts of solutes in a flood water can influence the residual oil content attainable by flooding a porous

TABLE 3. *Oil recoveries from static sand packs incubated for various times at 37 C*

Pack No.	Final State	Incubation Time	Oil as Per Cent of Pore Volume		
			Initial	Released	Residual
		Weeks			
13	Medium A, inoculated	1	32	5.3	27
14		2	27	5.5	21
15		3	30	8.8	21
16		4	28	4.1	24
17		5	33	4.0	29
18		6	26	5.2	21
Mean			29	5.5	24
19	Medium C, inoculated	1	32	4.0	28
20		2	36	3.8	32
21		3	23	5.8	18
22		4	31	6.9	24
23		5	38	7.1	31
24		6	35	7.6	27
Mean			33	5.2	27
25	0.1 per cent formalin in distilled water, sterile	1	32	5.5	27
26		2	35	6.3	29
27		3	33	5.0	29
28		4	29	4.9	25
29		5	33	4.4	29
30		6	43	5.3	38
Mean			34	4.3	30
31	Medium C, heat sterile	1	30	1.9	28
32		2	32	7.9	24
33		3	27	8.4	18
34		4	30	6.5	24
35		5	29	5.3	24
36		6	31	6.2	25
Mean			30	6.0	24

Sand: 35–45 mesh Ottawa. Oil: Belridge, California, crude, 15° API.

oil-containing medium. Only in the case of packs 7 and 8 is there any evidence which could be interpreted as demonstrating bacterial oil release. Here the inoculated sample had a lower residual oil content than any of the other packs, as well as a lower initial oil content. It is possible that the difference was caused by this variation in initial oil content. Also the medium used was almost free of sulfates, containing only the traces of sulfate contributed by the inoculum and the yeast extract. It, therefore, seems doubtful that sulfate-reducing bacteria could have promoted oil release in this pack.

Experiment 3 (table 3) was conducted with 24 sand packs divided into 4 groups of 6 packs each. Each pack in a group was treated the same except that incubation times varied. At 7-day intervals, one pack of each group was flooded out and released oil measured. Growth of

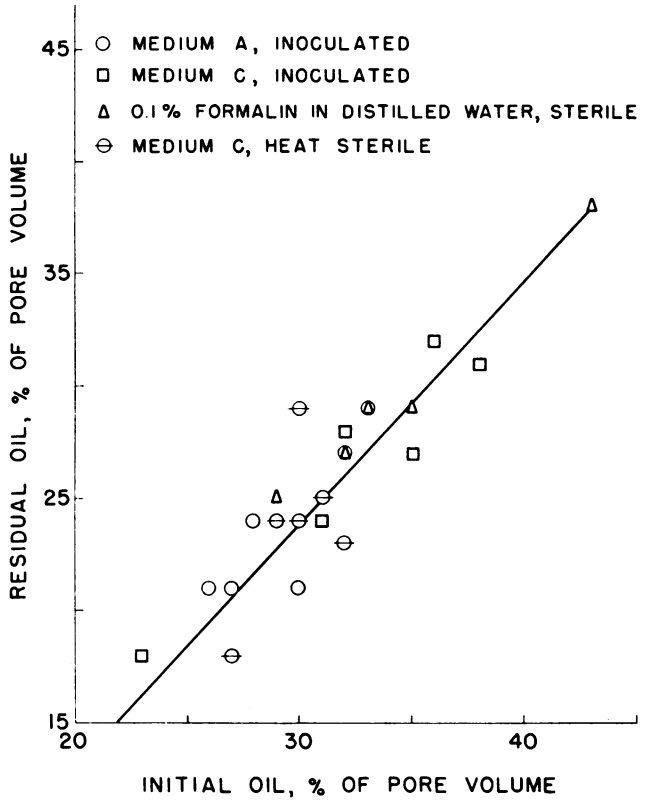

FIG. 1. The effect of initial oil content of sand packs on residual oil.

Desulfovibrio was rapid and vigorous in the inoculated packs because of the heavy inoculum and the optimum incubation temperature (37 C). All formaldehyde-sterilized controls remained sterile, but tests showed that the heat-sterilized controls had all become contaminated with bacteria other than *Desulfovibrio*.

A study of table 3 reveals differences in initial, released, and residual oil among the 24 sand packs, but the differences do not appear to be noticeably affected by the variables controlled in the four series of experiments. The mean values of residual oil for each of the four groups show a range of only 6 per cent, less than the range of individual values within each group, and seem to be correlated with the mean values of initial oil rather than with incubation time, presence or absence of bacterial action, or the aqueous medium used. A plot of residual oil versus initial oil for each of the 24 packs is presented in figure 1. It is evident that the residual oil content of a sand pack depends more on its initial oil content than on any of the variables controlled in this experiment. It can be deduced, therefore, that the experiment provides no evidence that *Desulfovibrio* bacteria influence the release of oil from sand by water flooding.

Experiment 4 (table 4) investigated three new variables: (1) different cultures of *Desulfovibrio*, (2) a calcareous porous medium (20-60 mesh marble chips), and (3) density of the crude oil. Medium AY was used

TABLE 4. *Oil recoveries from static packs of marble chips incubated for 50 days at 30 C*

Pack No.	Final Contents	Oil as per cent of Pore Volume		
		Initial	Released	Residual
1	Sterile, 1 per cent formalin, medium AY	20	<0.1	20
2		16	<0.17	16
3	Heat sterile, medium AY	13	<0.1	13
4		17	<0.1	17
5	Inoculated, 314-3, medium AY	23	<0.1	23
6		22	<0.1	22
7	Inoculated, garden soil, medium AY	19	<0.1	19
8		20	<0.1	20
9	Inoculated, S S P #6, medium AY	18	<0.1	18
10		18	<0.1	18
11	Inoculated, all stock cultures, medium AY	22	<0.1	22
12		20	<0.1	20

Marble: chips, 20–60 mesh. Oil: Healdton County, Oklahoma, crude, 24° API.

in all aqueous media including the formalin-sterilized controls. All packs were run in duplicate.

Again the two heat-sterilized controls became contaminated with bacteria other than *Desulfovibrio*. There was almost no oil release (<0.2 per cent) from any of the packs by the terminal flood after 50 days' incubation at 30 C, although growth of Desulfovibrio was vigorous in each of the four pairs of inoculated packs. Apparently the initial flood before incubation had reduced the residual oil content to the minimum value attainable with a medium AY flood, and none of the four enrichment cultures of Desulfovibrio was able to reduce the residual oil further. One of the cultures of Desulfovibrio was a mixture of all our available stock cultures (38).

Experiment 5 (table 5) was carried out with 20–60 mesh sand instead of crushed marble, but the inocula and methods were the same as for experiment 4. The results were also similar. Oil release was very slight (<0.6 per cent of the pore volume) in every case. Four of the five heat-sterilized controls, but none of the 5 formalin-sterilized controls, became contaminated, again with bacteria other than *Desulfovibrio*. The inoculated packs again showed vigorous growth of *Desulfovibrio*. The residual oil data were remarkably uniform; none of the treatments, heat sterilization, formaldehyde sterilization, and four different enrichment cultures of *Desulfovibrio*, had any effect on the residual oil content.

Experiment 6 (table 6) is of interest because of two innovations in technique: 1) The use of a successful new aseptic method of preparing heat sterilized con-

TABLE 5. *Oil recoveries from static sand packs incubated for 35 days at 30 C*

Pack No.	Final Content	Oil as Per Cent of Pore Volume		
		Initial	Released	Residual
13	Heat sterile—medium AY	17	<0.1	17
14		15	0	15
15		18	<0.3	18
16		20	<0.1	20
17		20	<0.1	20
18	Sterile—1 per cent formalin in	18	<0.1	18
19	medium AY	18	0	18
20		18	<0.1	18
21		20	0.6	19
22	Inoculated, 314-3, medium AY	18	<0.1	18
23		19	<0.1	19
24	Inoculated, S S P #6, medium	18	<0.1	18
25	AY	18	<0.1	18
26	Inoculated—soil—medium AY	17	<0.1	17
28		18	<0.1	18
29	Inoculated, all stocks, medium	20	<0.1	20
30	AY	20	<0.1	20

Sand: 20–60 mesh Ottawa. Oil: Healdton County, Oklahoma, 24° API.

TABLE 6. *Oil recoveries from static packs of oyster shell and sand using a new method of preparing heat-sterilized controls after 46 days' incubation at 30 C*

Pack No.	Final Contents	Oil as Per Cent of Pore Volume		
		Initial	Released	Residual
Sand				
1	Sterile, 1 per cent formaldehyde in medium AY	71	20	51
2	Sterile by heat, medium AY	68	18	50
3	Sterile, 1 per cent formaldehyde in medium AY	68	20	48
4	Inoculated, medium AY	68	11	57
5	Inoculated, medium AY	65	8.8	57
6	Sterile by heat, medium AY	71	5.0	66
Oyster				
7	Sterile by heat, medium AY	63	2.3	61
8	Sterile by heat, medium AY	73	7.3	66
9	Sterile, 1 per cent formaldehyde in medium AY	73	32	42
10	Sterile, 1 per cent formaldehyde in medium AY	61	14	47
11	Inoculated, medium AY	61	18	43
12	Inoculated, medium AY	59	16	44

Sand and Oyster: 20–60 mesh. Oil: Wilmington, California, Crude 15° API.

trols; and 2) the use of packs containing a measured volume of oil known to be greater than the residual oil after flooding.

Six packs were prepared with 20-60 mesh sand, and six, otherwise identical, with 20-60 mesh crushed oyster shell. The lower (tapered) end of the packed glass tube was connected to a "T" tube by a short length of rubber vacuum tubing. One arm of the "T" tube led through a rubber tube to a graduated separatory funnel filled with aqueous medium, and the other arm led to a graduated separatory funnel filled with oil. The top of the packed glass tube was connected through rubber vacuum tubing to a glass tube leading through a one-hole rubber stopper into a suction flask. The side arm of the suction flask was attached to a cotton filter through a short piece of vacuum tubing. Pinch clamps were placed on the rubber tubes in order to control the flow of the various fluids, and the separatory funnels were partially filled with oil and aqueous medium respectively, and cotton plugs were inserted. The entire assemblage was autoclaved. After cooling, the system was evacuated as completely as possible, and the core was saturated with a measured volume of heat sterilized aqueous medium. A measured volume of oil was then introduced, and spread throughout the pack by flooding with a determined volume of aqueous medium. The inoculum introduced at this point, was a mixture of nine *Desulfovibrio* cultures from ZoBell's collection, and included several alleged hydrocarbon-oxidizing strains (*Desulfovibrio halohydrocarbonoclasticus.*) The rubber tubes attached to the sand pack were then clamped off, and left in place during the incubation. The *Desulfovibrio* cultures grew well in the inoculated packs. After 46 days' incubation at 30 C, the terminal flood yielded considerable oil from all packs, since the packs had not been previously flooded. The residual oil content of five of the six pairs of packs varied by 5 per cent of the pore volume or less. The sixth pair showed a variation of 16 per cent. The maximum variation among all 12 packs was 14 per cent for the initial oil content, and 24 per cent for the residual oil content. In the sand packs the mean residual oil content of the inoculated packs (57 per cent) was greater than that of the sterile packs (54 per cent), thus providing no evidence for bacterial oil release. In the oyster shell packs, however, the mean residual oil content of the inoculated packs (43.5 per cent) was less than that of the sterile packs (54 per cent). The differences of 10.5 per cent is, however, less than the maximum difference between sterile oyster shell packs (19 per cent). It is, therefore, not possible to attribute the difference between inoculated packs and controls to bacterial oil release because of the large variations caused by unknown factors in the experimental procedures used.

Experiment 7 (table 7) was carried out in much the same manner as experiment 6.

The additional variable of three uninoculated, heat sterilized packs of each material, sand and oyster shell,

TABLE 7. *Oil recoveries from static sand and oyster shell packs containing Desulfovibrio or detergents after 49 days' incubation at 30 C*

Pack No.	Final Contents	Oil as Per Cent of Pore Volume		
		Initial	Released	Residual
Sand				
1	Heat sterile	75	58	18
2	Heat sterile	75	44	31
3	Sterile, 0.1 per cent formalin	71	46	25
4	Inoculated, all Scripps cultures	71	40	31
5	0.1 per cent alconox	68	40	28
6	0.1 per cent aerosol	63	46	17
7	0.1 per cent dreft	71	48	23
Oyster				
8	Heat sterile	58	29	29
9	Heat sterile	61	32	29
10	Inoculated, all Scripps cultures	69	44	25
11	Sterile, 0.1 per cent formalin	61	32	29
12	0.1 per cent alconox	65	32	32
13	0.1 per cent aerosol	65	41	24
14	0.1 per cent dreft	65	43	22

Sand or oyster shell: 20–60 mesh. Oil: 75 per cent Talco, Texas, crude, 25 per cent n-cetane. Detergents tested: 0.1 per cent dreft, 0.1 per cent aerosol, 0.1 per cent alconox.

treated with three different detergents was included. Again the *Desulfovibrio* inoculum was a mixture of nine of ZoBell's cultures. Again there were no differences in residual oil content after the terminal flood which were greater than those which might be attributed to random variations. The fact that the residual oil content of the packs is lower in this experiment than in experiment 6 may be attributed to the lower viscosity of the oil used, as may be inferred from its higher API gravity.

Experiment 8 (table 8) differed from the others in that a single long sand pack (9′5¼″) was used instead of many short packs. A piece of 1¼″ ID Saran pipe was drilled along its length with 5/32″ holes one foot apart and the holes were fitted with rubber stoppers. The tube was then mounted vertically, and a stainless steel screen sand retainer and a one hole rubber stopper carrying a glass delivery tube was attached to the bottom end. A short rubber tube and screw clamp was attached to the glass delivery tube. The entire system was then filled with 5 per cent phenol solution, and the system was allowed to stand overnight to sterilize all inner parts. A sterile one hole rubber stopper connected through a glass tube to a sterile cotton air filter was then placed on the upper end, and the phenol solution was drained out.

The system was then washed out with several volumes of sterile water to remove all phenol, and allowed to dry. Dry heat-sterilized sand was then packed into the tube, a sterile stainless steel screen sand retainer was placed in the upper end, and the pack was saturated

TABLE 8. *Oil recoveries from sections of long sand pack incubated for a total of 81 days at room temperature*

Section No.*	Length of Section	Time that Sulfate Reducing Bacteria Were Active	Oil as Per Cent of Pore Volume		
			Initial	Released	Residual
	inches	*days*			
1	14⅞	0	38	8.1	30
2	12⅛	0	29	5.1	24
3	11⅞	0–6	73	9	64
4	12	6–12	36	10	26
5	12	12–28	39	17	22
6	12	28–40	38	16	22
7	11⅞	40–48	44	21	23
8	12⅛	48–60	43	26	22
9 and 10	14⅜	60–81	54	21	29

Sand: 35–45 mesh Ottawa. Oil: Talco, Texas, crude, 23° API.

* In the initial flood, medium was injected into Section 1, which was at the bottom end of the vertical pack. Inoculation was performed at the end of Section 10. The sulfate-reducing bacteria migrated only as far as Section 3.

in sequence with CO_2, sterile aqueous medium, and oil, and then flooded with the same aqueous medium, in the conventional manner. After flooding for 20 days to remove excess oil, the pack was inoculated at the end nearest hole 10, and the pack was incubated horizontally at room temperature for 81 days.

Every 2 to 4 days each hole in the pack was sampled aseptically using a sterile hypodermic syringe. About 0.1 ml of fluid was withdrawn at each station. Counts for Desulfovibrio were performed on these samples by a cultural method involving colony counts in deep tubes of medium AY containing reduced iron powder to insure anaerobic conditions.

After 81 days' incubation, the bacteria had penetrated approximately 70 per cent of the pack. The pack was then sawed into sections, approximately 1 foot long, and each section was water flooded separately with 4 liters of sterile medium. Produced oil was measured, and then residual oil was measured by extracting all the oil from the sand with 1 liter of carbon tetrachloride, and measuring the infrared energy absorbed by the carbon tetrachloride extract at the wavelength of the carbon-hydrogen fundamental.

The *Desulfovibrio* grew well, and advanced from the inoculated end, section 10, through the pack at the almost uniform rate of 1.0 inches per day. No Desulfovibrio reached sections 1 and 2 of the pack, but there was some growth of contaminants in these sections.

The oil recovered by the terminal water flood plus the residual oil obtained by infrared analysis amounted to 89 per cent of the calculated initial volume of oil at the time of inoculation. This suggests that carbon tetrachloride extracts nearly all the oil remaining after the terminal flood.

The initial oil saturation of each section is calculated

TABLE 9. *Oil recoveries from long vertical sand packs after incubation for 100 days at room temperature under intermittent flooding from the bottom end*

Pack No.	Contents	Oil as Per Cent of Pore Volume		
		Initial	Released	Residual
1	Sterile—treated with 3 pore volumes of 1 per cent formalin in medium C	39	5.0	34
2	Inoculated, with 3 pore volumes of medium C containing 60 ml of culture 314-3 per liter	36	3.3	33
3	Sterile—treated with 3 pore volumes of 1 per cent formalin in medium A	36	7.9	29
4	Inoculated, with 3 pore volumes of medium A containing 60 ml of culture 314-3 per liter	34	4.8	29

Sand: 20–45 mesh Ottawa. Oil: Talco, Texas, crude 23° API.

TABLE 10. *Oil recoveries from outcrop cores of Woodbine sand after incubation for 39 days at room temperature under intermittent flood with aqueous media*

Sample No.	Contents	Initial Oil	Released Oil	Released Oil per cent of Initial
		grams	*grams*	
1 1 in diam, 2 5/16 in long	Medium A, inoculated with 2 pore volumes of 314-3	2.14	0.04	1.8
2 1 in diam, 2 5/16 in long	Medium A, sterile—no disinfectants	1.62	0.12	7.4

Source of plugs: Sandstone outcrop, Woodbine, Tarrant County, Texas. Characteristics of plugs: Bulk Vol. 29.8 ml; specific permeability to air 1280 millidarcies Oil: Healdton County, Oklahoma, 24° API. Flooding conditions: Intermittent flood, 10 ml/day.

from the sum of the released oil and residual oil. A rather large fluctuation in initial oil saturation is evident, with a trend toward lower values at the uninoculated end of the pack. The oil release figures show the same general trend. The residual oil saturation values are, with the exception of one nonconcordant result for section 3, remarkably uniform. Thus, while there is no apparent effect of *Desulfovibrio* on residual oil, the released oil shows an increase with increasing time of action of *Desulfovibrio* on each section of the pack. If it were established that the sections of the long sand pack behaved in the same manner as the small packs in experiment 3, where the residual oil content was proportional to the initial oil content, the lack of such a correlation in the data from this long sand pack would constitute evidence of bacterial oil release. Unfortunately, because of marked differences in the chemical nature, API gravity, and viscosity of the crude oils used in the two experiments, and because of differences in the techniques used in flooding the packs in the two experiments it cannot be assumed that the same relationships hold. Therefore, while the data suggest that bacterial oil release is operating in experiment 8, they do not constitute positive proof.

The API gravity of the original crude and the crude recovered from each section of the pack by the terminal flood was determined. The variations were very slight (<2°API). All of the recovered oils had a lower API gravity than the original oil, probably due to evaporation during recovery. Thus there is no evidence that the bacteria had any effect on the crude oil.

Tests on Sand Packs under Flowing Conditions

Experiment 9 (table 9) was conducted with large Lucite tubes (1½″ by 31½″) mounted vertically and packed with clean sand. After saturating the packs in the usual manner, and flooding out from the bottom up with 5 liters (17 pore volumes) of sterile medium, 1 liter (3.3 pore volumes) of medium containing the inoculum or germicide was passed through. An intermittent flood of 5 ml (about 0.5 inches) for each working day was carried out with the sterile medium for the inoculated packs, and with the germicide-treated medium for the sterile packs, during the incubation period of 100 days. The flooding process resulted in gradual oil release from all packs over a 100-day incubation period. The *Desulfovibrio* bacteria grew well in both inoculated packs. However, the released and residual oil values showed no significant differences between inoculated and sterile packs.

Tests on Natural Cores

The two experiments with natural cores, 10 and 11, (tables 10 and 11) were carried out with 1 inch diameter plugs in rubber sleeve permeameters using the same general methods as for most of the sand-pack experiments. The cores were saturated with nutrient medium, flushed with oil, and then flooded with many pore volumes of nutrient medium. They were then inoculated and incubated, then again flooded, and oil recovery measured.

Apparently in every case the pre-incubation flood had already recovered the essentially maximum recoverable oil, as only an insignificant volume of oil was ever recovered by the second flood. Thus the bacteria, although they always grew well and penetrated the cores, were unable to bring about any reduction in the residual oil content of the cores.

Tests Using ZoBell's Bottle Culture Method

To reinvestigate the original method used by ZoBell to demonstrate oil release by bacterial action, two ex-

periments were performed using synthetic oil "sands" in 125 ml glass-stoppered bottles. In the first experiment, samples of sand, marble chips, and crushed oyster shell (all 20-60 mesh) were mixed with Talco, Texas; Belridge, California; Fuhrman-Maschu, Texas; or Wilmington, California, crude oils and placed in 125-ml glass-stoppered bottles. One series was prepared by filling these bottles with Medium AY and inoculating with all the stock cultures (more than 30) in the belief that the environmental conditions would favor the growth of any hydrocarbon-oxidizing strain which might be present. Two series of sterile controls were prepared with 1 per cent formalin and 1 per cent mercuric chloride. These bottles, 30 in number, were allowed to incubate at 30 C for 85 days. No visible amounts of oil were released from any of these synthetic materials.

The second experiment of this type was more extensive (used a total of 138 bottles) and had an additional purpose—that of determining whether any of the cultures utilized the hydrocarbon materials. Samples of sand or crushed oyster shell (20-60 mesh) were mixed with Talco, Belridge, or Fuhrman-Maschu crude oils or with n-cetane (52 g of sand and 2 g of oil, or 36 g of oyster shell and 2 g of oil per bottle) and placed in 125-ml bottles. Different series were prepared using three different media: 1) medium C, 2) medium CY, 3) medium AY, and using three cultures of *Desulfovibrio* as inocula: 1) all cultures received from ZoBell (a mixture of 9 cultures of Desulfovibrio); 2) creek water; and 3) 314-3 from old sand pack. One series of controls was prepared with media, base materials and inocula to determine the amount of hydrogen sulfide which would be produced from the media in the absence of oil. Two series of sterile controls were prepared using 1 per cent formalin or 1 per cent mercuric chloride. After 64 days' incubation at 30 C, all cultures were examined for released oil and sulfide titrations were performed using potentiometric titration with silver nitrate. Only insignificant traces of oil were released. In medium C none of the 24 cultures containing oil showed appreciably more hydrogen sulfide than controls without oil. In medium AY, 2 out of 24 showed over 80 mg per liter more hydrogen sulfide in the presence of oil than in its absence, but both produced less sulfide than one of the controls, and therefore are not regarded as significant. In medium CY, 2 cultures of the 24 showed more sulfide than any of the controls. The experiment suggests that medium CY is superior to the others employed for demonstrating hydrocarbon oxidation by sulfate-reducing bacteria. Later experiments therefore employed a similar medium, that is a dilute solution of yeast extract in a complete mineral salts medium.

Production of surface active agents is one mechanism suggested by which bacteria might assist in the release of oil from oil bearing formations. To test the effect of surface-active agents on release of oil in the types of apparatus used in these studies, two experiments, using 20 bottles each, were conducted in 125-ml glass-stoppered bottles containing synthetic oil "sands". Samples of sand and oyster shell (20-60 mesh) wet with Talco crude oil in the proportions given in the preceding experiment, were placed in 125-ml glass-stoppered bottles. The base medium used was sterile medium A containing 0.05 per cent yeast extract. From this the following solutions to be tested were prepared:

	pH
1. 1 per cent Alconox	9.2
2. 1 per cent Aerosol	5.0
3. 1 per cent Twitchell Reagent	8.0
4. 1 per cent Alconox plus 1 per cent formalin	9.2
5. 1 per cent Aerosol plus 1 per cent formalin	5.0
6. 1 per cent Twitchell plus 1 per cent formalin	6.5

The pH of these solutions was not adjusted to neutral. None of the samples was inoculated. Sterile controls were prepared with 1 per cent formalin and 1 per cent mercuric chloride. The solutions were placed in the bottles containing oil "sands" and allowed to incubate undisturbed for 42 days. During this period no oil was released. They were then inverted several times and allowed to incubate 10 more days. Alconox alone then gave good oil release. Those maintained sterile with formalin gave the same results as the others. Since agitation of the sand would not occur in nature, the value of this method of observation is doubtful.

TABLE 11. *Oil recoveries from plugs of Wilcox sand after 42 days' incubation at room temperature under intermittent flood with aqueous media*

Sample No.	Contents	Treatment	Oil Content, Volume		
			Initial	Released	Residual
			ml	*ml*	*ml*
1 1 in diam, 1⅛ in long	Medium A, sterile by heat	Incubated under flowing conditions	0.3	0.0	0.3
2 1 in diam, 1 in long	Medium A, inoculated with 1 pore volume of "FW"	Incubated under flowing conditions	0.4	0.0	0.4
3 1 in diam, 1$\frac{1}{32}$ in long	Medium A, inoculated with 1 pore volume of "FW"	Incubated in bottle	0.3	0.0	0.3
4 1 in diam, 1⅛ long	Medium A, sterile by heat	Incubated in bottle	0.4	0.0	0.4

Source of plugs: Cores from Wilcox Formation, Chesterville Field, Texas. Characteristics of plugs: Bulk Vol. 12.8 ml. Effective permeability to medium A at end of incubation period 38 to 582 md. Oil: Fuhrman-Maschu, Texas, crude 28° API.

TABLE 12. *Hydrogen sulfide titers of Desulfovibrio cultures incubated for 34 days at 37 C in the presence and absence of Talco, Texas, crude oil*

Inoculum	Source	Oil	H_2S	Difference
			mg/l	*mg/l*
C-7	California water	0	172	
C-7	California water	Present	83	−89
C-8	California water	0	0.8	
C-8	California water	Present	81	80
C-9	California water	0	35	
C-9	California water	Present	94	59
C-10	California water	0	156	
C-10	California water	Present	23	−133
WT-6	West Texas water	0	32	
WT-6	West Texas water	Present	2.4	−30
WT-7	West Texas water	0	145	
WT-7	West Texas water	Present	46	−99
WT-8	West Texas water	0	41	
WT-8	West Texas water	Present	23	−18
3 pure culture	California water	0	27	
3 pure culture	California water	Present	29	2
6 pure culture	Soil	0	36	
6 pure culture	Soil	Present	26	−10

Another experiment was set up in the same manner as the one previously described except that the pH of the solutions was adjusted to the neutral range. Results were essentially the same as recorded above.

Utilization of Hydrocarbons by Desulfovibrio

Since no organisms are known which can oxidize hydrocarbons anaerobically in the absence of oxidized sulfur or nitrogen compounds capable of serving as hydrogen acceptors, any anaerobic growth in a sulfate-containing medium free of such other hydrogen acceptors, and containing a hydrocarbon as the sole energy source, is assumed to be the result of the action of sulfate-reducing bacteria. Hydrocarbon utilization in such a medium may be established by several analytical methods:

1. By measuring the decrease in the amount of hydrocarbon after incubation.
2. By determining total hydrogen sulfide content at intervals, since any oxidation of hydrocarbon must be accompanied by sulfate-reduction.
3. By determining sulfate at intervals.

The second method has been used more frequently in these studies than the other methods. Hydrogen sulfide was determined by the sensitive potentiometric method described in the section on experimental methods.

Experiment 12 was performed with 38-ml glass-stoppered bottles containing 5 ml of Fuhrman-Maschu, Texas crude oil; one ml of a natural oil field water or a pure culture of *Desulfovibrio* as an inoculum, and filled with medium M1. After 16 days' incubation at 37 C, those cultures which failed to show growth of *Desulfovibrio* (seven) were discarded, and those which grew (nine) were transferred again using 1 ml inocula,

TABLE 13. *Hydrogen sulfide titers of Desulfovibrio cultures incubated for 58 days at 30 C in the presence and absence of Talco, Texas, crude and n-hexadecane*

Culture	Source	mg/l H_2S in Presence of:		
		Cetane	Talco crude	No oil
314-3	Marine sediments	21.3	29.8	25.5
Flood H_2O	Darst Creek flood water	0	38.3	59.6
HC 29:137:4	Marine sediments	29.8	127.7	119.2
HC 29:136:3	Marine sediments	329.9	327.8	166.0
HC 29:136:4	Marine sediments	68.1	166.0	229.9
HC 29:136:5	Marine sediments	92.8	76.6	166.0
HC 29:P130:2	Marine sediments	59.6	46.8	166.0
30	Marine sediments	47.7	29.8	114.9
11	Marine sediments	170.3	102.1	144.8
All Scripps cultures	Marine sediments	191.6	144.8	153.3
GP #10	California water sample	204.4	166.0	195.8
Magnolia W. Tex. #7	Texas water sample	143.1	161.8	166.0
Magnolia S. Tex. #1	Texas water sample	197.5	102.1	114.9
GP #7	California water sample	285.3	234.1	255.5
GP #9	California water sample	38.3	93.7	55.9
GP #8	California water sample	323.6	327.8	255.6
Nodule	From Lisbon unit strainer	17.0	29.8	55.3
Sterile		0	0	0

into fresh medium and Talco, Texas oil. After 34 days' incubation at 37 C, all cultures were titrated potentiometrically. Table 12 presents the data obtained in this manner. Six of the 9 cultures tested gave more sulfide in the absence of Talco crude oil than in its presence while the differences for the other 3 cultures can hardly be regarded as significant, as will be explained in the discussion of experiment 13.

The experiment just outlined contains a possible source of error. Improper fit of the standard-taper glass stopper in each bottle might allow sufficient gaseous interchange with the air to allow some of the produced hydrogen sulfide to be lost to the atmosphere or to be oxidized by incoming oxygen, or the incoming oxygen might be sufficient to provide for some hydrocarbon oxidation by aerobic bacteria. In event of the latter occurrence, sulfate-reducing bacteria, unable to utilize hydrocarbons, could perhaps utilize the oxidation products produced by aerobic hydrocarbon-oxidizing bacteria, thus giving an erroneous impression that the sulfate-reducing bacteria were able to oxidize hydrocarbons. All work in the literature where enrichment cultures were used is open to this criticism; only one paper (Rosenfeld, 1947) deals with this question, and

even here there is doubt as to the purity of the *Desulfovibrio* cultures used.

Experiment 13 (table 13) with 17 inoculum cultures of *Desulfovibrio* eliminated this source of error. Glass ampoules 145 mm long by 20 mm outside diameter with a 3-inch entry tube of 6 mm tubing containing 20 ml of medium CX and either 0.02 g of Talco crude oil, n-cetane, or no hydrocarbon were inoculated with one ml of *Desulfovibrio* culture. Sterile controls were prepared for each series with 1 per cent formalin. After filling and inoculating, the ampoules were evacuated, flushed with nitrogen, sealed with a flame and shaken at 30 C for 58 days. Sulfide determinations were made on all three series.

If a culture produces significantly more sulfide in the presence of oil than it does in the same medium in the absence of oil, this is evidence of hydrocarbon oxidation by *Desulfovibrio*. However, it is difficult to determine how great the difference must be to be significant. The presence of oil modifies the physiochemical environment of the bacteria, and may influence the sulfide concentration even though it is not utilized by the bacteria.

A study of table 13 is of assistance in making a decision as to the difference in sulfide titers to be taken as significant. Two of the cultures, HC 29:136:3 and GP 8, produced more sulfide from either n-cetane or Talco crude than any of the others (98 to 164 mg per liter difference). Other differences recorded, for those cultures giving more sulfide in the presence of oil, were: For Talco crude, 4 and 38 mg per liter; for n-cetane, 25, 39, 8, 83, and 29 mg per liter. Only the figure of 83 mg per liter approaches the values for the two most outstanding cultures. This culture, S. Texas 1, will be considered as showing utilization of n-cetane but not Talco crude. For screening purposes a difference of more than 80 mg per liter of hydrogen sulfide will be accepted as evidence of oil utilization, provided that the culture containing oil also gives a higher sulfide titer than any of the controls without oil. Most of the cultures produced more H_2S in the absence of oil than in its presence.

Cultures HC 29:136:3 and GP 8, the only ones which showed evidence of utilization of both cetane and crude oil, were subcultured into mineral medium C containing 0.001 g per liter of methylene blue and 154 g per liter of emulsified n-hexadecane or of Talco crude oil. The cultures grown in hexadecane medium were used in 1 ml quantities to inoculate the hexadecane medium, and the crude oil cultures were subcultured to crude oil medium. Cultures were grown in 96 ml of medium in sealed glass ampoules under nitrogen. After 59 days' incubation at 30 C, the methylene blue was only partly decolorized in the n-hexadecane cultures, but completely decolorized in the crude oil cultures. Tests showed the presence of no sulfide in any of these cultures. Thus we were unable to confirm that these cultures could oxidize hydrocarbons anaerobically.

TABLE 14. *Hydrogen sulfide titers of Desulfovibrio cultures incubated for 88 days at 30 C in the presence and absence of a mixture of Talco, Texas, crude oil and n-hexadecane*

Sample No.	Source of Inoculum	mg/l H_2S in Presence of:	
		No oil	Crude oil-hexadecane mixture
1	Tar sand	0	0
2	Fresh water sediment	64	132*
3	Soil	64	0
4	Fresh water sediment	222	158
5		119	247*
6	Soil	77	184*
7	Mud from oil sump pit	230	111
8		0	60*
9		30	247*
10		17	0
11		0	302*
12		47	26
13		51	179*
14		68	0
15		30	158*
16		13	0
17		119	115
18	Oil sand core	0	0
19		0	0
20		0	0
21		0	0
22		0	0
23		0	0
24		0	0
25		0	0
26		30	0
27		0	0
28	Lake sediment and water	209	315*
29		13	166*
30	Slough water	60	9
31	Creek sediment	119	158*
32	Water from oil well	111	0
33		158	0
34		0	0
35		179	94
36		0	0
37		0	0
38	Water from oil well	0	0
39		0	0
40		0	0
41		0	0
42		0	0
43		17	0
44		34	0
45		21	0
46		0	0
47		72	0
48	Sterile control	0	0

* Samples producing more H_2S in the presence of oil than in the absence of oil.

Another experiment was performed with 48 samples of water and sediments, most of them collected from petroliferous areas in Canada where it was believed to

TABLE 15. *Hydrogen sulfide titers of Desulfovibrio cultures incubated for 81 days at 30 C in the presence and absence of a mixture of Talco, Texas, crude oil and n-hexadecane*

Sample No.	mg/l H_2S in Presence of:	
	No oil	Crude oil n-hexadecane mixture
2	17	0
5	38	64*
6	23	21
8	33	32
9	34	43*
11	43	37
13	77	95*
15	85	85
28	47	26
29	55	38
31	13	29*
Sterile control	0	0

* Samples producing more H_2S in the presence of oil than in the absence of oil.

be likely that environmental conditions would favor the development of hydrocarbon-oxidizing *Desulfovibrio*. One gram of each sample was inoculated into each of two 30-ml glass stoppered-bottles. One bottle of each pair was filled with medium M2, and the other with medium M2 containing 5 ml per bottle of a mixture of 1 volume of n-hexadecane with 3 volumes of Talco, Texas crude oil. After 88 days' incubation at 30 C, sulfide titrations were performed, by the potentiometric method described, on each bottle. Table 14 presents the data.

The limit of accuracy of the titrations is approximately ±5 mg of H_2S per liter. Therefore, cases in which the H_2S concentration in the bottle containing oil exceeds that in the bottle not containing oil by five mg per liter or more are considered to be possible cases of utilization of oil by sulfate-reducing bacteria. Table 14 reveals 11 such cases. One ml of each of these 11 samples, marked by an asterisk in table 14, was transferred from the oil-containing bottle to a fresh 30 ml bottle of the same oil-containing medium. After 8 days' incubation at 30 C, one ml of each of these cultures was transferred to each of two ampoules of approximately 100 ml capacity. One ampoule of each pair was nearly filled with medium M3, and the other with medium M3 plus 15 ml of the same oil mixture as used in the preceding experiment. The cultures were evacuated, filled with nitrogen, sealed in a flame, and incubated for 81 days at 30 C, and were then titrated as before. Table 15 presents the results. It will be noted that 4 of the 11 inocula produced more H_2S in the absence of oil than in the presence of oil, 3 inocula produced essentially the same amount of H_2S in the absence and presence of oil, and 4 produced more H_2S in the presence of oil than in its absence. In only one case is the sulfide produced in the presence of oil higher than the maximum value of the controls without oil. Even in this case, the sulfide value is only 10 mg per liter above the maximum control titer.

It is not possible to decide, on the basis of these experiments, whether the few isolated cases in which enrichment cultures of *Desulfovibrio* produced more sulfide from dilute yeast extract medium plus oil than from

TABLE 16. *Summary of conditions and apparatus used in oil release experiments*

Exp. No.	Size of Pack or Core		Type of Porous Medium	Particle Size Range	Type of Oil	API Gravity of Oil	Nutrient Media Used	Sterilizing Agent for Control	Inoculum	Vol of Inoculum, Pore Vol or ml	Effective Permeability to Water, Oil in Place, Darcys	Specific Gas Permeability Darcys
	Diam	Length										
	in	*in*		*mesh*								
1	1⅛	11¼	Ottawa sand	35–45	Belridge, California	14	CM-1	Sat. $HgCl_2$	314-3	1 p.v.	18–80	
2	⅞	9½	Ottawa sand	35–45	Wilmington, California	15	Many media*	Heat	314-3	5 ml	9–18	
3	⅞	9½	Ottawa sand	35–45	Belridge, California	15	Many media*	Heat, HCHO	314-3	1 p.v.	8–28	
4	⅞	9½	Marble chips	20–60	J. A. Smalley, Okla.	24	AY	Heat, HCHO	All stock cultures	1 p.v.	—	
5	⅞	9½	Ottawa sand	20–60	Oklahoma	24	AY	Heat, HCHO	All stock cultures	1 p.v.	—	
6	⅞	9½	Ottawa & oyster	20–60	Wilmington, California	15	AY	Heat, HCHO	All ZoBell cultures	1 p.v.	—	
7	⅞	9½	Ottawa & oyster	20–60	Talco, cetane	30	AY	Heat, HCHO	All ZoBell cultures	1 p.v.	—	
8	1¼	114	Ottawa sand	35–45	Talco, Texas	23	CM-1	Phenol	314-3	25 ml	15–78	
9	1½	31½	Ottawa sand	20–45	Talco	23	A	HCHO	314-3	3 p.v.	7–14	
10	1	2 5/16	Sand core	?	Oklahoma	24	A	Heat	314-3	2 p.v.		1.28
11	1	1	Sand core	?	Fuhrman-Maschu, Texas	28	A	Heat	Flood water	1 p.v.	0.04–0.58	

* All variations on medium C—see tables 9 and 10.

dilute yeast extract medium alone represent hydrocarbon oxidation by *Desulfovibrio*. Miller (1949) reported that Desulfovibrio cultures grown on lactate in the presence of small amounts of yeast extract can produce up to 2500 mg per liter of H_2S in 3 days. The rates of sulfide production and the amounts of sulfide produced in our experiments with hexadecane or crude oil were so low that they could have been caused by the utilization of traces of non-hydrocarbon impurities in the oils, or by some side reaction such as the oxidation of a trace of hydrocarbon to fatty acids by some organism other than *Desulfovibrio*, using traces of dissolved oxygen, or some other hydrogen acceptor present in the yeast extract or other constituents of the medium, followed by oxidation of the fatty acid by *Desulfovibrio* at the expense of sulfate reduction. Many different media and growth conditions have been employed without providing unequivocal evidence of the oxidation of hydrocarbon substrates by *Desulfovibrio*. In our opinion definite proof of the ability of sulfate-reducing bacteria to oxidize petroleum hydrocarbons is not to be found in the literature. It is hoped that further research will enable the publication of conclusive quantitative data to settle this point.

Discussion

The mechanisms by which bacteria may release oil from porous rocks should be considered in connection with our knowledge of the physiology of sulfate-reducing bacteria. These bacteria do not produce large amounts of acids, gases or surface-active agents. It seems unlikely that the quantities of these products, which might be produced by sulfate-reducing bacteria, would affect oil recovery to a measurable extent. Our results agree with those of Beck (1947) and Kuznetsov (1950) in that sulfate-reducing bacteria attack crude oil extremely slowly and incompletely, if at all. This finding is consistent with the fact that several of the mechanisms advanced by ZoBell to explain oil release by sulfate-reducing bacteria depend on the ability of sulfate-reducing bacteria to utilize crude oil as an energy source and to modify it chemically.

Although sulfate-reducing bacteria probably act on petroleum too slowly to be of practical value in oil recovery, it is recognized that there is evidence for an important role played by bacteria in the migration and accumulation of oil throughout geological time (ZoBell, 1952), and sulfate reducers are probably of importance among the bacteria concerned.

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Summary

Factors investigated in this study include: 1) Sand versus lime material in packs; 2) different strains of *Desulfovibrio* (6 enrichment cultures, some being mixtures of several, comprising a total of 38 cultures of *Desulfovibrio* from marine sediments, including several hydrocarbon-oxidizing strains obtained from ZoBell, oil well brines from Texas, California, and Illinois, cores and creek mud from Texas); 3) types of oils (5 crude oils and 1 n-cetane-crude oil mixture): 4) many different aqueous media compositions; 5) two different methods of saturating the packs; 6) different volumes of inocula and various methods of inoculating the packs; 7) both static and flowing conditions during incubation. Eleven separate experiments, involving the use of 103 oil-containing unconsolidated packs of sand or calcareous material and six sandstone core samples, yielded negative results, in that no increase in oil recovery could be brought about by the growth of sulfate-reducing bacteria within the porous medium.

Several of the mechanisms by which sulfate-reducing bacteria may release oil depend wholly or in part on the ability of the bacteria to utilize crude oil as an energy source, or to modify the crude oil. Studies on the oxidation of crude oil and n-hexadecane showed little or no utilization of these substances by any of the *Desulfovibrio* cultures available. It does not seem likely that sulfate-reducing bacteria can modify crude oil sufficiently to affect oil recovery in commercial quantities.

It is concluded that the results of the tests reported herein have been sufficient in scope to render it improbable that presently known strains of *Desulfovibrio* can be applied successfully in the field for the secondary recovery of oil. This conclusion does not preclude the possibility that a strain of *Desulfovibrio* could be found which will release oil from porous media under certain conditions.

References

Beck, J. V. 1947 Penn grade progress on use of bacteria for releasing oil from sands. Producers Monthly, **11**, 13–19.

Breed, R. S., Murray, E. G. D., and Hitchens, A. P. 1948 *Bergey's Manual of Determinative Bacteriology*, 6th Edition, The Williams & Wilkins Co., Baltimore.

Faragher, W. F., Morrell, J. C., and Comay, S. 1929

Interaction of alkyl sulfides and salts of mercury, J. Am. Chem. Soc., **51**, 2774–2781.

KUZNETSOV, S. I. 1950 Investigation of the possibility of contemporaneous formation of methane in gas-petroleum formations in the Saratov and Buguruslan regions. Mikrobiologiya, **19**, 193–202. (In Russian).

MACKENZIE, K. 1952 The metabolism of *Vibrio desulfuricans* in anaerobic petroliferous formations. Biochem. J., **51**, xxiv–xxv.

MILLER, L. P. 1949 Rapid formation of high concentrations of hydrogen sulfide by sulfate-reducing bacteria. Contribs. Boyce Thompson Inst., **15**, 437–465.

O'BRYAN, O. D., AND LING, T. D. 1949 The effect of the bacteria, *Vibrio desulfuricans* on the permeability of limestone cores. Texas J. of Sci., **1**, 117–128.

ROSENFELD, W. D. 1947 Anaerobic oxidation of hydrocarbons by sulfate-reducing bacteria. J. Bacteriol., **54**, 664–665.

TAMELE, M. W., AND RYLAND, L. B. 1936 Potentiometric determination of mercaptans. Ind. Eng. Chem., Anal. Ed., **8**, 16–19.

UPDEGRAFF, D. M., AND WREN, G. B. 1953 Secondary recovery of petroleum oil by *Desulfovibrio*. U. S. Patent No. 2,660,550. Assigned to Socony-Vacuum Oil Company, Incorporated.

ZOBELL, C. E. 1946a Functions of bacteria in the formation and accumulation of petroleum. Oil Weekly, **120**, 30–36.

ZOBELL, C. E. 1946b Bacteriological process for treatment of fluid-bearing earth formations. U. S. Patent No. 2,413,278.

ZOBELL, C. E. 1947a Bacterial release of oil from sedimentary materials. Oil and Gas J., **46**, No. 13, 62–65.

ZOBELL, C. E. 1947b Bacterial release of oil from oil-bearing materials. World Oil, Part 1, **126**, No. 13, 36–44. Part 2, **127**, No. 1, 35–40.

ZOBELL, C. E. 1952 Part played by bacteria in petroleum formation. J. Sediment. Petrol., **22**, 42–49.

ZOBELL, C. E. 1953 Recovery of hydrocarbons. U. S. Patent No. 2,641,566. Assigned to the Texaco Development Corporation.

Studies on the Efficiencies of Disinfectants for Use on Inanimate Objects[1]

II. Relative Activities on Porous Surfaces

R. L. STEDMAN, E. KRAVITZ AND H. BELL

Industrial Test Laboratory, Philadelphia Naval Shipyard, Naval Base, Philadelphia, Pennsylvania

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Part I of this study (Stedman, Kravitz, and Bell, 1954) was devoted to a survey of the relative antimicrobial activities of disinfectants on a nonporous (stainless steel) surface using a new performance test method. Since a wide variety of surfaces having different compositions are encountered in practice, it was decided to extend the survey to other representative surfaces. This report summarizes the findings obtained on two porous surfaces (battleship linoleum and asphalt tile) using a test procedure similar in most respects to that employed in Part I.

MATERIALS AND METHODS

Except for the carriers, the same experimental method described in Part I was used in this study. Briefly, the method consists of contaminating the upper surfaces of one-inch square carriers with a mixture of *Micrococcus pyogenes* var. *aureus* cells, *Salmonella schottmuelleri* cells, and *Trichophyton interdigitale* spores with or without normal horse serum, air-drying this inoculum, disinfecting the upper surfaces of the carriers with various dilutions of disinfectant for 10 minutes at 20 C, recovering the survivors by differential culturing, and calculating the percentage survivals of the various species.

In this study, two types of carriers were employed; battleship linoleum and asphalt tile. Microscopic inspection of the surfaces of the carriers indicated a difference in the degree of porosity. Using low (75×) magnification, the surface of the battleship linoleum was observed to consist of numerous, adjacent, irregularly-shaped pores of small widths and diameters. The surface of the asphalt tile consisted of "patches" of pores surrounded by nonporous material; most of the pores on the tile were much larger than those of linoleum and of irregular widths and diameter. The battleship linoleum conformed to Federal Specification LLL-L-351a; the asphalt tile conformed to Federal Specification SS-3-306.

All squares were sterilized by ultraviolet irradiation

[1] The opinions expressed herein are those of the authors and not necessarily similar to the views of the Department of the Navy.